Mechanisms of T-Lymphocyte Accumulation during Experimental Pleural Infection Induced by *Mycobacterium bovis* BCG[▽]

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Tuberculous pleurisy is a frequent extrapulmonary manifestation characterized by accumulation of fluid and inflammatory cells in the pleural space. Here, we investigated the mechanisms of T-lymphocyte accumulation in the pleural space by using a murine model of pleurisy induced by *Mycobacterium bovis* BCG. Intrathoracic (i.t.) injection of BCG (4.5×10^5 bacteria/cavity) induced accumulation of T lymphocytes in the pleural cavities of C57BL/6 mice. We observed the presence of CFU in pleural washes conducted 1, 2, 3, 7, and 15 days after pleurisy induction. Pretreatment with fucoidan inhibited T-lymphocyte accumulation at 1 day, but not at 15 days, after BCG-induced pleurisy. Accordingly, adoptive transfer of fluorescein isothiocyanate-labeled blood mononuclear cells to infected mice showed that T lymphocytes migrated into the pleural cavity 1 day (but not 15 days) after BCG injection. Cell-free pleural wash fluids recovered from mice 1 day after BCG i.t. stimulation (day 1 BCG-PW), but not day 7 or day 15 BCG-PW, induced in vitro T-cell transmigration, which was dependent on L-, P-, and E-selectins. In contrast, day 7 BCG-PW (but not day 1 BCG-PW) induced in vitro T-lymphocyte proliferation via interleukin-2 (IL-2) and gamma interferon (IFN-γ). Accordingly, in vivo IL-2 or IFN-γ neutralization abolished T-lymphocyte accumulation 7 days after pleurisy induction. Our results demonstrate that pleural infection induced by BCG leads to T-lymphocyte accumulation in two waves. The acute phase depends on selectin-mediated migration, while the second wave of T-lymphocyte accumulation seems to depend on a local proliferation induced by cytokines produced in situ.

Tuberculosis remains one of the leading infectious causes of mortality in the world, with approximately one-third of the population infected with mycobacteria (http://www.who.int/mediacentre/factsheets/fs104/en/). Pleural tuberculosis is the most common form of extrapulmonary tuberculosis and is one of the most relevant clinical pleural problems, characterized by accumulation of fluid and inflammatory cells in the pleural space, which occurs through a mechanism that is still not completely understood (13).

The pleural space is a restricted environment that consists of a metabolically active monolayer of mesothelial cells involved in the maintenance of homeostasis, which becomes activated during pleural inflammation (4, 12). Pleural mesothelial cells are able to initiate and regulate an innate response to *Mycobacterium* species infection by stimulating cytokine production as well as cell accumulation in the pleural cavity (12, 18, 19, 21). Accumulation of inflammatory cells and T lymphocytes in pleural exudate is often observed as much in patients with tuberculous pleurisy (14) as in experimental infection (17, 20, 23, 30). It is also known that pleural effusion derived from mycobacterial infection presents high numbers of type 1 cytokine producer lymphocytes in pleural fluid (13). Even though the role of T lymphocytes in the immunopathogenesis of tuberculosis is well established, the mechanisms by which these cells accumulate and survive in the pleural effusion are not fully understood.

There are two different mechanisms by which T lymphocytes accumulate at inflammatory sites: migration (2) and local proliferation (8, 10). Lymphocyte migration into inflammatory sites is facilitated by differential expression of adhesion molecules and homing receptors on T cells. Type 1 differentiated T lymphocytes upregulate, among other molecules, selectins and selectin ligands, which are related to increased migration of T cells into inflammatory sites (28). However, accumulation of T lymphocytes in nonsecondary lymphoid organs can also occur through a mechanism that is independent of active migration and instead relies on local activation and proliferation (8, 15).

In the present study, we investigate the mechanism by which T lymphocytes accumulate during mycobacterium infection, using a murine experimental model of pleurisy induced by BCG.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (15 to 20 g) were provided by the Oswaldo Cruz Foundation (Fiocruz, Brazil) Breeding Unit (Rio de Janeiro, Brazil). Animals were caged with free access to food and fresh water in a room with a temperature ranging from 22 to 24°C under a 12-h light/dark cycle at Farmanguinhos Experimental Animal Facility. All experimental procedures were performed according to guidelines provided by the Committee on Ethical Use of Laboratory Animals of the Oswaldo Cruz Foundation.

BCG-induced pleurisy. *Mycobacterium bovis* BCG was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with Middlebrook ADC (Difco). Pleurisy was induced by an intrathoracic (i.t.) injection of 100 μl of 4.5×10^5 CFU of BCG at middle-log growth phase, as previously described (17). Control animals received an equal volume of sterile saline. Mice were sacrificed by excess amounts of CO_2, and their thoracic cavities were washed with 1 ml of RPMI 1640 and 10 mM EDTA (Sigma-Aldrich, St. Louis, MO). Total leukocyte counts were determined with an automated particle counter (Beckman Coulter, Inc., Fullerton, CA). A Cytospin (Thermo Shandon, Waltham, MA) smear was performed

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with staining by the May-Grunwald-Giemsa method for differential leukocyte counts under light microscopy (100× lens objective; Olympus). For CFU determination, cells recovered from pleural cavities were subjected to decontamination by the Petroff method before culture in Lowenstein-Jensen medium (31).

Immunofluorescence staining and flow cytometric analysis. For flow cytometric analysis, cells recovered from pleural cavities were processed as described below. Samples of 10^6 cells were incubated in phosphate-buffered saline (PBS) plus 10% rat serum and 0.1% sodium azide (PBS-S; Sigma-Aldrich) and FcγIIR block monoclonal antibodies (MAbs) at 1:100 (CD16/CD32; BD Pharmingen, San Diego, CA) for 30 min at 4°C to avoid nonspecific background staining. After being blocked, cells were labeled with the appropriate concentration of MAb conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE; BD Pharmingen) diluted in PBS-S, and incubated for another 30 min at 4°C. After being labeled, cells were washed and resuspended in PBS-0.1% sodium azide for data acquisition with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). For intracellular c-Fos analysis, cells were first fixed with 4% paraformaldehyde (Sigma-Aldrich), and CD3 molecules were stained as described above. Cells were permeabilized in PBS-S–0.1% saponin (Sigma-Aldrich), incubated with rabbit anti-mouse c-Fos antibodies (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), and then stained with secondary FITC-conjugated anti-rabbit immunoglobulin G (IgG; Sigma-Aldrich). Apoptotic T lymphocytes were evaluated by concomitant detection of CD3 and 7-AAD staining. Briefly, after being blocked, cells were incubated with PBS-S containing 20 μg/ml of 7-AAD and anti-mouse CD3-PE for 30 min at 4°C. Cells were then resuspended in 4% paraformaldehyde containing 20 μg/ml of actinomycin for analysis. Forward scatter (FSC) and side scatter (SSC) parameters were set to exclude dead cells, and at least 10^4 lymphocytes were analyzed per sample. Control staining for determination of the positive population was performed based on an irrelevant IgG isotype labeled with FITC or PE. Once determined, quadrants were rigorously maintained for all analyses. Data analyses were performed using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

Determination of cytokine production by ELISA. To evaluate interleukin-2 (IL-2) production in pleural wash fluids recovered after BCG-induced pleurisy, a standard sandwich enzyme-linked immunosorbent assay (ELISA) was performed in accordance with the manufacturer's instructions (BD Pharmingen). For production of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), or IL-12 p40 by nonrestimulated leukocytes recovered from saline- or BCG-injected mice (at 1, 7, and 15 days postinfection), cell ELISA was performed as previously described (5). The plates were read at 490 nm on a Spectramax 190 (Molecular Devices).

Animal treatment. Fucoidan, a lectin-carbohydrate blocker (Sigma-Aldrich), was diluted in sterile saline and intravenously (i.v.) administered (10 mg/kg of body weight) 1 h before and 7 and 14 days after a single i.t. injection of BCG. Anti-mouse IL-2 (20 μg/cavity; Pharmingen), anti-mouse IFN-γ (20 μg/cavity; R&D), or control (20 μg/cavity) MAb was administered i.t. 4 days after BCG-induced pleurisy. At the indicated time points, the pleural cavities of the mice were washed and lymphocytes were evaluated by flow cytometry as described above.

Transwell migration assay. For transmigration assays, pleural wash fluids recovered from mice injected i.t. with saline ($n = 10$) or BCG ($n = 15$) were pooled after the thoracic cavities were washed with 500 μl of saline. The pleural wash fluids were centrifuged ($420 \times g$ for 10 min at 20°C), and saline and BCG cell-free supernatants (hereinafter referred to as SPW and BCG-PW, respectively) were recovered and kept at −80°C until use. Migration assays were performed with 24-well Transwell plates (Corning Costar) with 5-μm-pore polycarbonate filters. Lymphocytes were enriched in a T-cell enrichment column (R&D Systems) and resuspended at a density of 3×10^6 cells in assay buffer (Hanks balanced salt solution without Ca^{2+}/Mg^{2+}, containing 30 mM HEPES, 0.25% bovine serum albumin, pH 7.4). A cell suspension (200 μl) was placed in the upper chamber, and 400 μl of assay buffer or stimulus (SPW or BCG-PW) was placed in the lower chamber. After incubation for 4 h at 37°C, cells recovered from the lower chamber were counted, labeled as described above, and analyzed using a flow cytometer.

Transendothelial migration assay. A murine thymic endothelioma cell line (tEnd.1) was kindly provided by Thereza Christina Barja Fidalgo (Universidade do Estado do Rio de Janeiro, Brazil). These cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum containing 25 μg/ml of gentamicin. Adherent cells were detached by washing them with PBS, followed by a 5-min incubation in a trypsin-EDTA solution at 37°C. tEnd.1 cells (2×10^5) were cultured in Transwell polycarbonate culture inserts in 24-well culture plates (5.0-μm pore diameter; BD Falcon, San Diego, CA) and allowed to grow to confluence for 48 h (37°C and 5% CO_2). Transwell inserts containing cultured monolayers were washed once in warm PBS and were added to 24-well culture

plates containing 500 μl of SPW, BCG-PW, or buffer. tEnd.1 monolayers were incubated with fucoidan (25 μg/ml), anti-CD62P MAb (25 μg/ml), anti-CD62E MAb (25 μg/ml), or isotype control MAb (25 μg/ml). Splenocytes were pretreated with anti-CD62L MAb (25 μg/ml), fucoidan (25 μg/ml), or isotype control MAb (25 μg/ml). Pretreatments were performed 30 min prior to the assay. Splenocytes (3×10^6 cells/well in 200 μl of RPMI 1640) were added to the upper chamber of the Transwell tissue culture inserts, and the plate was incubated for 4 h at 37°C throughout the duration of the transmigration assay. Transmigrated cells were collected from the lower chamber, counted, and stained with antibodies against CD3, as described above.

Peripheral blood mononuclear labeling and adoptive transfer. Peripheral blood cells were subjected to double-density gradient centrifugation by the Histopaque-1119 and Histopaque-1077 (Sigma-Aldrich) methods to recover peripheral blood mononuclear cells. Cells were washed with sterile PBS and incubated with a 0.05-mg/ml solution of FITC (Sigma-Aldrich) for 30 min at 4°C. Cells were then washed, resuspended in sterile saline, and adoptively transferred into injected mice by an i.v. injection (4.5×10^5 cells/mouse) (3).

One hour after adoptive transfer, recipient mice received an i.t. injection of BCG or saline, and after 24 h, the FITC-labeled T lymphocytes recovered from the pleura were evaluated by flow cytometry (FACSCalibur). To evaluate the late lymphocyte accumulation, donor and recipient mice received an i.t. injection of BCG. On the 13th and 14th days after i.t. stimulation, recipient mice received FITC-labeled leukocytes recovered from donor mice on the 13th and 14th days, respectively. FITC-labeled T cells were set in the T-lymphocyte region determined by an FSC and SSC dot plot and confirmed by positive CD3 fluorescence staining.

Evaluation of proliferation. Splenocytes from normal C57BL/6 mice were incubated with 100 μl of BCG-PW or SPW recovered from mice 7 days after i.t. injection (hereinafter referred to as day 7 BCG-PW or SPW, respectively) in the presence of one of the following MAbs (20-μg/ml final concentration) (29): anti-mouse IL-2, anti-mouse IL-12 p40, anti-mouse TNF-α, and anti-mouse IFN-γ (R&D). Cell proliferation was evaluated using a CytoLite kit (Packard PerkinElmer, Wellesley, MA). Briefly, cells were incubated with solutions provided by the manufacturer, and proliferation was evaluated by determining counts per second of luminescence with Top Count NXT (Packard).

In vivo incorporation of BrdU. Lymphoproliferative cell numbers were determined as extensively described previously (24). Briefly, mice were injected intraperitoneally daily with bromodeoxyuridine (BrdU) (1 mg/200 μl of PBS per mouse), starting on the day of BCG infection and continuing for up to 7 days. The pleural cavities were then washed, and cells were stained with CD3-PE as described above. Cells were fixed and permeabilized with 4% paraformaldehyde-1% saponin and then frozen in 10% dimethyl sulfoxide and 90% fetal bovine serum to permeabilize the nuclear membrane overnight. Cells were then thawed, washed, resuspended in freshly prepared DNase I (4 units DNase/ml; Invitrogen) diluted in Hanks balanced salt solution with Ca^{2+} and Mg^{2+}, and incubated in a 37°C water bath for 1 h. Following DNase treatment, mouse anti-BrdU was added, followed by FITC-conjugated anti-mouse IgG. Cells were than washed and acquired with a FACSCalibur flow cytometer as described above.

Statistical analysis. Statistical significance was assessed by analysis of variance followed by the Newman Keuls t test. Results are expressed as means ± standard errors of the means (SEM), and the significance level in all cases was set at P values of <0.05.

RESULTS

Kinetics of lymphocyte accumulation during BCG-induced pleurisy. Fig. 1A shows an initial peak of T-lymphocyte accumulation 1 day after i.t. injection of BCG, followed by a significant decrease in cell numbers within 48 h. After this time point, T-lymphocyte accumulation started to increase again until 15 days after BCG injection. Similar profiles were observed for $CD4^+$ and $CD8^+$ T-lymphocyte accumulation (Fig. 1B and C). It is interesting to note that $\gamma\delta^+$ T-lymphocyte accumulation in the pleural cavities of BCG-infected mice was significantly different from that for the control mice between 1 and 15 days after BCG i.t. injection, despite a small decrease of $\gamma\delta^+$ T lymphocytes at 48 h (Fig. 1D). Concerning T-lymphocyte decrease 48 h after BCG infection, the numbers of apoptotic cells in the pleural cavities of
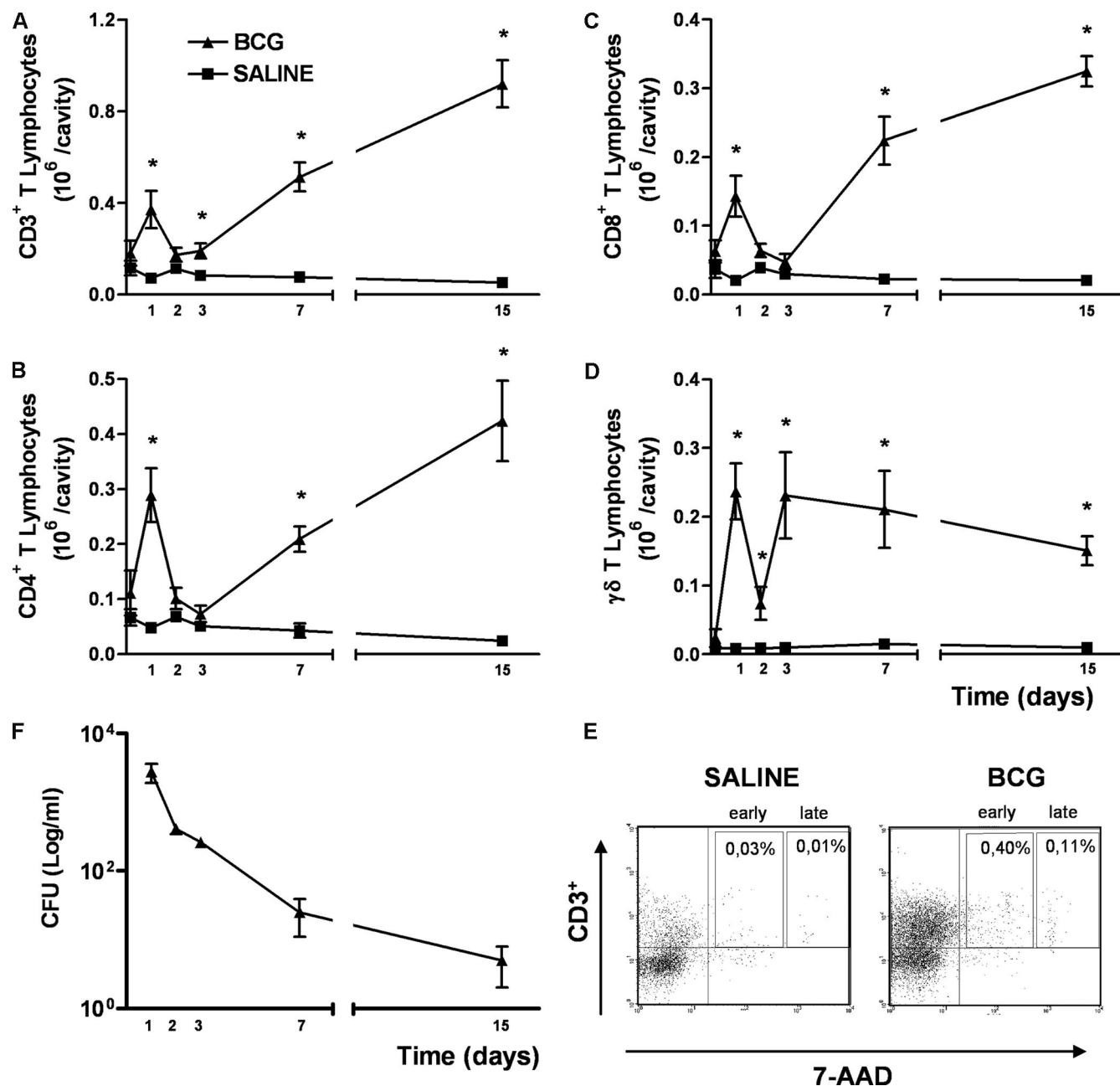
FIG. 1. Kinetics of CD3$^+$ T-lymphocyte (A), CD4$^+$ lymphocyte (B), CD8$^+$ lymphocyte (C), and γδ T-cell (D) accumulation and BCG growth curves (F) after i.t. injection of saline (■) or BCG (▲). Mice received an i.t. injection of BCG, and the pleural cavity was washed at the indicated time points. (E) Flow cytometry analysis of CD3$^+$ cells stained with 7-AAD 36 h after i.t. injection of BCG or saline. Percentage values of positively stained cells from one representative animal per group are shown in dot plots. T lymphocytes were evaluated by flow cytometry as described in Materials and Methods. The T-lymphocyte region was determined with FSC and SSC dot plots and confirmed by positive CD3 fluorescence staining. Results are expressed as means ± SEM for groups of six animals each in three experiments. Statistically significant differences ($P < 0.05$) between control and BCG-stimulated groups are indicated by asterisks.

36-h-infected mice were evaluated. The representative dot plots demonstrate increases in numbers of T cells stained with 7-AAD 36 h after BCG i.t. injection relative to the numbers for the control group (Fig. 1E).

The lymphocyte numbers were in inverse proportion to the numbers of CFU detected in pleural wash fluids recovered from BCG-injected mice. As shown in Fig. 1F, high levels of

bacterial load were observed at 1 day, followed by decreases in colony counts until 15 days, after BCG i.t. injection.

Production of type 1 cytokines by leukocytes recovered from the pleural cavities of BCG-injected mice. Several studies have demonstrated the production of type 1 cytokines as well as their role in a pulmonary experimental model of *Mycobacterium* species infection (2). Figure 2 shows the evaluation of
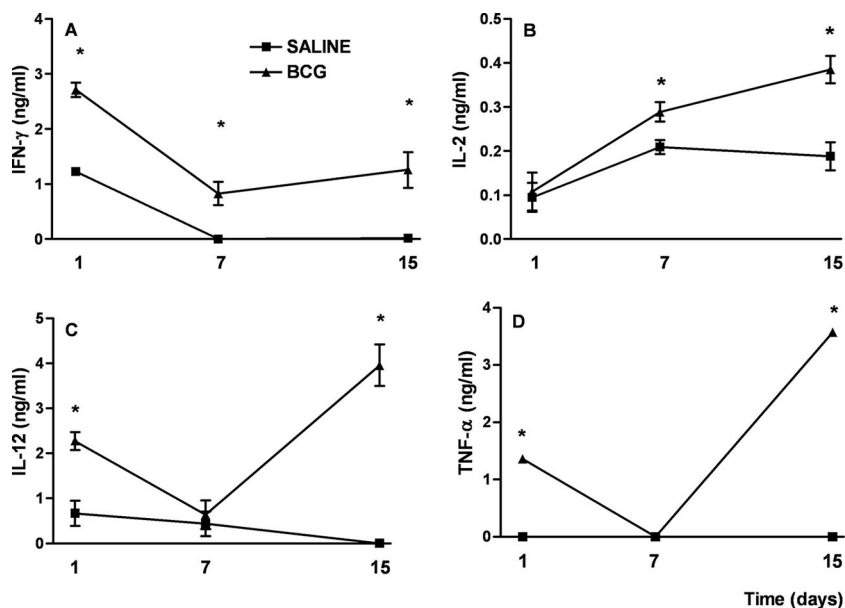
FIG. 2. Kinetics of IFN-γ (A), IL-2 (B), IL-12 p40 (C), and TNF-α (D) production by cultured total leukocytes recovered from the pleural cavity after i.t. injection of saline (■) or BCG (▲) at different time points. Mice received an i.t. injection of BCG, and the pleural cavity was washed at the indicated time points. Cytokine production was evaluated by ELISA as described in Materials and Methods. Results are expressed as means ± SEM for groups of at least six animals each in two different experiments. Statistically significant differences ($P < 0.05$) between control and BCG-stimulated groups are indicated by asterisks.

IFN-γ, TNF-α, IL-12 p40, and IL-2 levels in pleural exudates 1, 7, and 15 days after i.t. injection of BCG (Fig. 2). IFN-γ production was sustained from 1 until 15 days postinfection (Fig. 2A), whereas increased levels of IL-12 p40 and TNF-α were detected in supernatants of leukocytes recovered at 1 and 15 days postinfection but not in those of leukocytes recovered at 7 days postinfection (Fig. 2C and D). Interestingly, an increase in IL-2 production accompanied the infection's time course (Fig. 2B). Production of the type 2 cytokine IL-5 was also detected at 1 day, but not at 7 or 15 days, after BCG-induced pleurisy (data not shown).

Selectin blockade in BCG-injected mice. Selectins are crucial for leukocyte migration into inflammatory sites (15, 29). In this context, we pretreated mice with fucoidan, a blocker of lectin-carbohydrate interaction that is able to impair selectin-dependent cell migration (26). Blockade of lectin-carbohydrate interaction failed to inhibit total leukocyte migration 1 day after induction of pleurisy (Fig. 3A), even though it inhibited neutrophil migration to basal levels ($2.69 \times 10^6 \pm 0.35 \times 10^6$ [BCG] versus $1.43 \times 10^6 \pm 0.34 \times 10^6$ [fucoidan] neutrophils/cavity; $P < 0.05$) and inhibited T-lymphocyte accumulation in the pleural cavity 1 day after i.t. injection of BCG (Fig. 3B).

Selectin blockade for 15 days after BCG infection failed to inhibit total leukocyte accumulation in the pleural cavities of BCG-injected mice (Fig. 3A). Similarly, fucoidan treatment did not modify BCG-induced mononuclear cell accumulation ($5.1 \times 10^6 \pm 0.5 \times 10^6$ [BCG] versus $4.6 \times 10^6 \pm 0.6 \times 10^6$ [fucoidan] mononuclear cells/cavity; $P > 0.05$) or T-lymphocyte accumulation (Fig. 3B). In contrast, BCG-induced neutrophil influx into the pleural cavities of mice was significantly inhibited by fucoidan pretreatment 15 days after pleurisy induction ($1.95 \times 10^6 \pm 0.21 \times 10^6$ [BCG] versus $1.09 \times 10^6 \pm 0.1 \times 10^6$ [fucoidan] neutrophils/cavity; $P < 0.05$). To evaluate

whether fucoidan pretreatment was modifying T-lymphocyte activation, we measured cytokine production by leukocytes recovered from BCG-stimulated mice. Fucoidan treatment did not alter cytokine production by total leukocytes recovered from the pleural cavities of BCG-injected mice, as demonstrated by TNF-α, IL-12 p40, and IFN-γ detection 15 days after infection (Fig. 3C).

T-lymphocyte migration into the inflammatory site. We further investigated the abilities of BCG-PW and SPW recovered from i.t. injected mice to promote cell migration in vitro. Using a Transwell model, we observed that BCG-PW recovered 1 day after BCG injection was able to induce CD3$^+$ T-cell migration (Fig. 4A). In contrast, day 7 and 15 BCG-PW failed to induce in vitro T-cell chemotaxis, suggesting that the T-lymphocyte migration observed 7 and 15 days after BCG stimulation did not depend on local production of chemoattractant mediators. As an additional control, we spiked the SPW with BCG (3×10^3 CFU/well) and did not observe lymphocyte migration. We further investigated the involvement of L-, P-, and E-selectins in day 1 BCG-PW-induced T-cell migration by using a trans-endothelial assay. CD3$^+$ T lymphocytes migrated more strongly toward day 1 BCG-PW than to SPW or SPW spiked with BCG, a phenomenon that was inhibited by pretreatment of splenocytes with anti-L- and anti-P-selectin MAbs (Fig. 4B). CD3$^+$ T migration was also inhibited when endothelial cells were pretreated with anti-E-selectin MAb. It is worth noting that day 7 and 15 BCG-PW failed to induce CD3 T-lymphocyte migration throughout the endothelial cell monolayer, which was unchanged by preincubation of cells with anti-selectin MAbs (data not shown). Therefore, we performed an assay of adoptive transfer of FITC-labeled leukocytes into BCG- or saline-stimulated mice. Figure 4C shows that 1 day after BCG-stimulation, adoptively transferred T lymphocytes labeled with
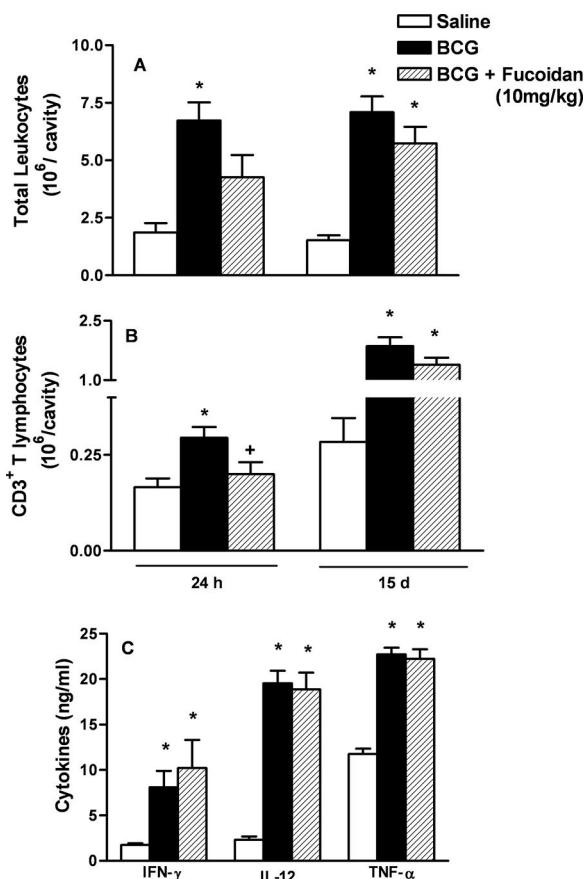
FIG. 3. Effect of fucoidan treatment (10 mg/kg i.v., 1 h before and 7 and 14 days after i.t. stimulation) on total leukocyte (A) and CD3$^+$ T-lymphocyte (B) accumulation in the mouse pleural cavity 1 day (left) and 15 days (right) after BCG-induced pleurisy. Total leukocytes were evaluated with an automated particle counter, while CD3$^+$ T lymphocytes were determined with an FSC and SSC dot plot and confirmed by positive CD3 fluorescence staining. The effect of fucoidan treatment on cytokine production in the mouse pleural cavity 15 days after BCG-induced pleurisy is shown in panel C. Cytokine production was evaluated by ELISA as described in Materials and Methods. Results are expressed as means ± SEM for groups of at least six animals each in four different experiments. Statistically significant differences ($P <$ 0.05) between control and BCG-stimulated groups are indicated by asterisks, and + represents differences between treated and untreated groups.
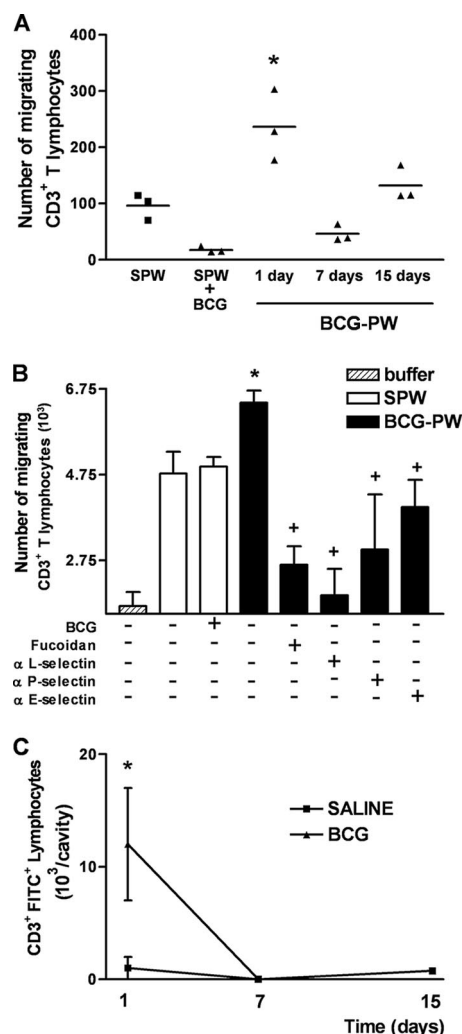

FIG. 4. (A) In vitro migration of enriched T lymphocytes toward SPW or BCG-PW recovered from mice i.t. injected 1 day, 7 days, or 15 days before. SPW spiked with BCG (3×10^3 CFU/well) was used as assay control. Spleen leukocytes were placed in the upper chamber of 5.0-μm-pore-diameter Transwell inserts, and cells that migrated into the bottom chamber were recovered and labeled for CD3. (B) In vitro migration of T lymphocytes toward day 1 SPW or BCG-PW. T cells were pretreated with anti-mouse-CD62L or anti-mouse-CD62P, while endothelial cells were pretreated with anti-mouse-CD62E. T cells and endothelial cells pretreated with the isotype control as well as SPW spiked with BCG (3×10^3 CFU/well) were used as an assay control. Cell migration was evaluated as described above. (C) Accumulation of FITC-labeled lymphocytes in the pleural cavities of BCG-injected mice. FITC-labeled T cells were set in the lymphocyte region, determined with an FSC and SSC dot plot, and confirmed by positive CD3 fluorescence staining. Results are expressed as means ± SEM for groups of at least five animals each, and each experiment was repeated twice. Statistically significant differences ($P <$ 0.05) between control and BCG-stimulated groups are indicated by asterisks, and + represents differences between treated and untreated groups.

FITC migrated into the mouse pleural cavity. However, 7 and 15 days after BCG-induced pleurisy, no differences were observed in FITC-labeled T-lymphocyte accumulation in the pleural cavities of BCG-injected and noninjected mice (Fig. 4C) ($P <$ 0.05). Since long-term experimental pleural tuberculosis is characterized by a continual granulocyte influx (17, 23), we verified the ability of labeled transferred granulocytes to migrate into the pleural cavity after BCG stimulus as an experimental control. Pleural granulocytes were determined by setting FSC and SSC gates, and accumulation was determined by increase of mean fluorescence intensity in the granulocyte's gate. We confirmed the presence of FITC-labeled granulocytes at 1 day (33.24 ± 1.3 [saline] versus 37.76 ± 0.8 [BCG] mean fluorescence intensity; $P <$ 0.05) and 15 days (25.8 ± 1.1

[saline] versus 32.3 ± 1.1 [BCG] mean fluorescence intensity; $P <$ 0.05) after BCG injection.

Role of IL-2 and IFN-γ in BCG-induced late lymphocyte accumulation. Since late T-lymphocyte accumulation was not a consequence of cell migration, we evaluated the ability of cytokines present in the pleural fluid to promote T-cell proliferation.
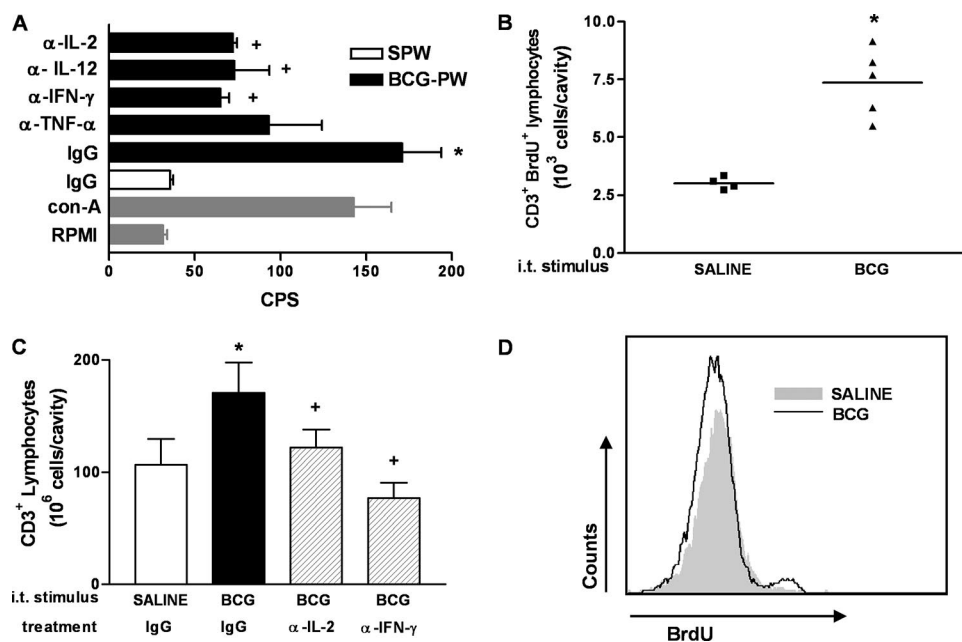
FIG. 5. Mechanism of late lymphocyte accumulation. (A) Splenocytes recovered from uninfected mice were cultured with day 7 BCG-PW (black bars) or SPW (white bars). Positive and negative controls are represented by gray bars. Proliferation was evaluated by luminescence as described in Materials and Methods. Experiments were repeated twice with pooled pleural washes of at least six animals per group, and the results are expressed as means ± SEM for at least six wells. Statistically significant differences ($P < 0.05$) between the SPW and BCG-PW plus control IgG groups are indicated by an asterisk, and + represents the differences between treated and untreated groups. CPS, counts per second. (B) BrdU incorporation in vivo. BrdU was administered daily for up to 7 days, and its incorporation by T cells was determined as described in Materials and Methods. ▲, BCG; ■, saline; *, $P < 0.05$. (C) IL-2 and IFN-γ neutralization in vivo. Anti-mouse IL-2 (20 μg/ml) or anti-mouse IFN-γ (20 μg/ml) MAb was administered as described in Materials and Methods. The lymphocyte region was determined with FSC and SSC dot plots and confirmed by positive CD3 fluorescence staining. Results are expressed as means ± SEM for groups of six animals each in two experiments. Statistically significant differences ($P < 0.05$) between control and BCG-stimulated groups are indicated by an asterisk, and + represents differences between treated and untreated groups. (D) Representative histogram shows BrdU detection in CD3+ cells in saline (shaded histograms) or BCG (bold lines). Representative histograms for one animal out of five per group are shown.

Splenocytes from normal animals were stimulated by day 7 BCG-PW or SPW. Splenocytes were incubated with BCG-PW or SPW either in the absence or in the presence of antibodies against different cytokines, and proliferation was evaluated by luminescence using a CytoLite kit. Figure 5A shows that BCG-PW was able to induce T-lymphocyte proliferation similarly to ConA. In contrast, SPW was a weak proliferative stimulus, comparable to the negative control (RPMI). Pretreatment with MAbs against IL-2, IFN-γ, or IL-12 p40 significantly inhibited the proliferative effect of BCG-PW, while treatment with MAbs against TNF-α did not impair T-lymphocyte proliferation. It is important to note that BCG-PW recovered from mice injected 1 day before did not induce T-lymphocyte proliferation (data not shown) and that the addition of isotype control MAb did not affect T-lymphocyte proliferation (Fig. 5A). In agreement, in vivo treatment with BrdU demonstrated high levels of lymphoproliferation through BrdU incorporation by CD3+ cells in mice that had received an i.t. injection of BCG 7 days before (Fig. 5B and D). These data were confirmed by in vivo IL-2 or IFN-γ neutralization prior to BCG stimulus, by inhibiting T-lymphocyte accumulation in the mouse pleural cavity 7 days after pleurisy induction (Fig. 5C). Accordingly, neither IL-2 nor IFN-γ induced T-lymphocyte migration in vitro (data not shown).

Evaluation of molecules involved in lymphocyte proliferation. A high rate of IL-2 in situ activates T cells and induces IL-2 receptor-dependent downstream pathways, resulting in c-Fos expression (33). Therefore, we analyzed the involvement of IL-2R/CD25 and c-Fos in the T-lymphocyte proliferative response. Figure 6A shows a significant increase in the expression of CD25 in pleural CD3+ T lymphocytes recovered 7 days after BCG i.t. stimulation (Fig. 6A and B). These cells also show an increase in c-Fos expression relative to the level for the control group (Fig. 6C and D).

DISCUSSION

In this study, we demonstrate the mechanisms of T-lymphocyte accumulation at different time points of the pleural inflammatory reaction induced by i.t. injection of BCG. The importance of T-lymphocyte accumulation at mycobacterium-infected sites is crucial in both mouse and human pulmonary tuberculoses. T cells comprise more than 70% of total leukocytes in tuberculous pleural effusions (32), in which CD4+ and CD8+ T cells are essential for effective control of *Mycobacterium tuberculosis* infection (6). We demonstrate increases of γδ+ T-lymphocyte numbers in the pleural cavities of mice that received an i.t. injection of BCG 1 day after infection, which remained augmented up to 15 days after infection. It is worth noting that this profile is different from the biphasic profiles observed for CD4+ and CD8+ T-lymphocyte accumulation in BCG-injected mice, in which T-cell numbers increased on day 1, returned to basal levels no later than day 2, and then in-
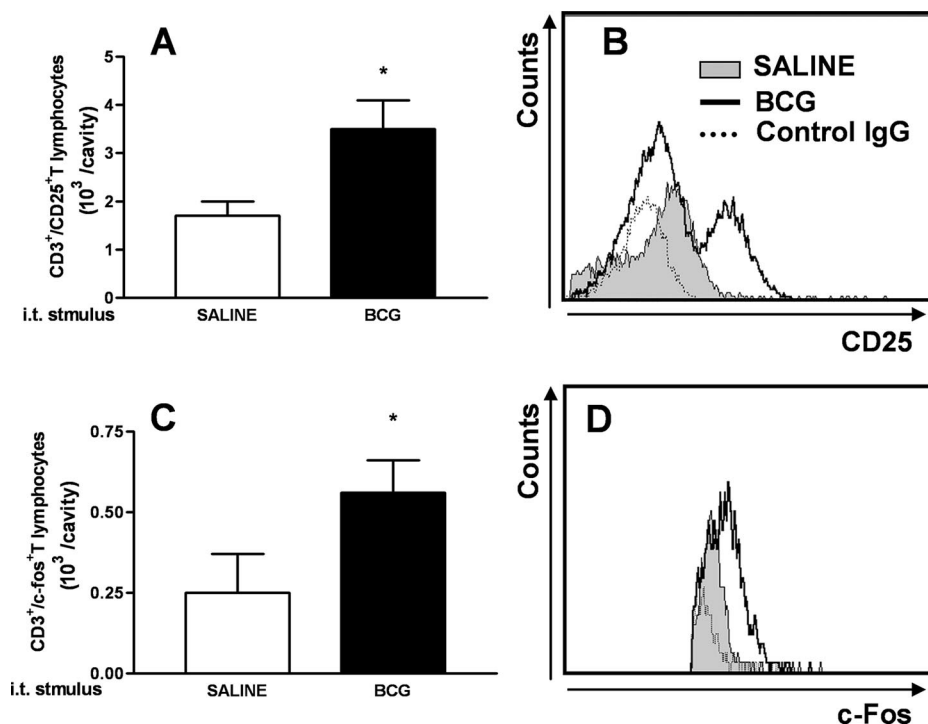
FIG. 6. CD25 and c-Fos expression in pleural T lymphocytes 7 days after BCG-induced pleurisy. CD25 (A, B) and c-Fos (C, D) expression levels were evaluated for $CD3^+$ T lymphocytes previously gated according to an FSC/SSC profile followed by positive CD3 fluorescence staining. Representative histograms demonstrate the increase in fluorescence provided by increase in CD25 (B) and c-Fos (D) expression in $CD3^+$ T lymphocytes recovered from the pleural cavities of mice 7 days after BCG i.t. injection. Results are expressed as means $\pm$ SEM for groups of at least six animals each in two different experiments. Statistically significant differences ($P < 0.05$) between control and BCG-stimulated group are indicated by asterisks.

creased from day 7 until day 15 postinfection. Recently, it was demonstrated that cells recovered from patients with pleural effusion due to tuberculosis infections are able either to undergo apoptosis or to proliferate when stimulated with mycobacterial antigens in vitro (9). It is worth noting that we observed an increase in T-cell death after BCG stimulation within 36 h, contributing to the decrease in T-cell numbers at 48 h.

Despite the fact that the role of T cells is well described for immune reactions induced by mycobacterium infection, the mechanisms by which these cells accumulate at the infectious sites have not yet been clarified, especially for tuberculous pleural effusions. We first analyzed whether T lymphocytes were recruited into the pleural site by the classical mechanism of leukocyte migration through adhesion molecules. The crucial role of selectins in inducing T-lymphocyte migration in other airway inflammatory models has been demonstrated (1); however, most studies concerning the role of selectin in mycobacterial infections are restricted to phenotyping T lymphocytes and, so far, do not provide evidence that such adhesion molecules are involved in T-lymphocyte migration and accumulation in pleural effusion (29).

The inhibition of selectin by fucoidan pretreatment diminished the acute accumulation of T lymphocytes in the pleural cavities of infected mice, suggesting that these cells are actively migrating to the inflammatory site. It is interesting to note that repeated treatment with fucoidan did not inhibit the T-lymphocyte numbers and cytokine production at 15 days postinfection, whereas the neutrophil migration observed at 15 days

postinfection was inhibited, demonstrating the efficacy of selectin blockade by fucoidan and suggesting that this treatment is not able to interfere with lymphocyte activation. These results were corroborated with in vitro transmigration assays using specific antibodies against L-, P-, or E-selectins and in vivo adoptive transference of lymphocytes that showed an active migration of T lymphocytes at 1 day but not at 15 days postinfection.

In fact, selectins seem to be involved in leukocyte migration during a mycobacterially induced immune response (16). Recently, using an experimental model of pulmonary tuberculosis, Schreiber and coworkers (25) demonstrated that although the initial lymphocyte accumulation in lung granuloma 21 days after *M. tuberculosis* infection is dependent on selectin ligands, chronic-phase cell-mediated immune responses depend not on selectins but probably on organized neolymphoid structures within the lungs.

In agreement with those findings, we also demonstrated that splenic T lymphocytes proliferate when cultured with pleural fluid from BCG-infected mice and that the addition of MAbs to neutralize IL-2 and IFN-γ impaired the lymphoproliferative response. However, such effects were not observed when splenocytes were cultured with BCG-PW and treated with MAbs to block TNF-α. Since IL-12 is not involved in lymphocyte proliferation but is involved in IFN-γ production, the lack of proliferation observed in splenocytes treated with anti-mouse-IL-12 represents an indirect effect of IFN-γ activity. In fact, such results are in accordance with the in vivo observa-

tions that pleural T cells incorporate BrdU at the site of infection and that both IL-2 and IFN-γ are necessary to induce T-lymphocyte accumulation during BCG-induced pleurisy in a mechanism that does not depend on T-cell migration. Accordingly, in vitro studies demonstrated that pleural mononuclear cells stimulated with *M. tuberculosis* proliferate in the presence of type 1 cytokines (9). Indeed, IFN-γ has been shown to induce T-cell proliferation (7). Such an effect relies on the density of IFN-γR2 expression on the T-cell membrane, a phenomenon modulated by the presence of growth factors such as IL-2 (22). Moreover, activation of IL-2β chain receptor on effector and memory T lymphocytes induces proliferation without the requirement of antigen presentation (11). Accordingly, T lymphocytes present in the mouse pleural cavities 15 days after BCG i.t. injection expressed high levels of IL-2α receptor (CD25), in addition to increased expression of the AP-1 component c-Fos, indicating a proliferative phenotype.

Taken together, our results demonstrate that T-lymphocyte accumulation at early time points after mycobacterial infection is due to migration from the bloodstream to the inflammatory site, while at late time points of BCG-induced pleurisy, T-lymphocyte accumulation depends not on migration but on local proliferation facilitated by IL-2 and IFN-γ present in the pleural cavity.

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REFERENCES

1. **Ainslie, M. P., C. A. McNulty, T. Huynh, F. A. Symon, and A. J. Wardlaw.** 2002. Characterization of adhesion receptors mediating lymphocyte adhesion to bronchial endothelium provides evidence for a distinct lung homing pathway. Thorax **57:**1054–1059.
2. **Allen, S. S., and D. N. McMurray.** 2003. Coordinate cytokine gene expression in vivo following induction of tuberculous pleurisy in guinea pigs. Infect. Immun. **71:**4271–4277.
3. **Andrade, W. N., M. G. Johnston, and J. B. Hay.** 1998. The relationship of blood lymphocytes to the recirculating lymphocyte pool. Blood **91:**1653–1661.
4. **Antony, V. B.** 2003. Immunological mechanisms in pleural disease. Eur. Respir. J. **21:**539–544.
5. **Beech, J. T., T. Bainbridge, and S. J. Thompson.** 1997. Incorporation of cells into an ELISA system enhances antigen-driven lymphokine detection. J. Immunol. Methods **205:**163–168.
6. **Bergroth, V., Y. T. Konttinen, D. Nordström, T. Pettersson, and E. Tolvanen.** 1987. Lymphocyte subpopulations, activation phenotypes, and spontaneous proliferation in tuberculous pleural effusions. Chest **91:**338–341.
7. **Bernabei, P., E. M. Coccia, L. Rigamonti, M. Bosticardo, G. Forni, S. Pestka, C. D. Krause, A. Battistini, and F. Novelli.** 2001. Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. J. Leukoc. Biol. **70:**950–960.
8. **Dai, Z., and F. G. Lakkis.** 2001. Secondary lymphoid organs are essential for maintaining the CD4, but not CD8, naive T cell pool. J. Immunol. **167:**6711–6715.
9. **Das, S. D., D. Subramanian, and C. Prabha.** 2004. Cell proliferation and apoptosis: dual-signal hypothesis tested in tuberculous pleuritis using mycobacterial antigens. FEMS Immunol. Med. Microbiol. **41:**85–92.
10. **Dieli, F., J. Ivanyi, P. Marsh, A. Williams, I. Naylor, G. Sireci, N. Caccamo, C. Di Sano, and A. Salerno.** 2003. Characterization of lung γδ T cells following intranasal infection with *Mycobacterium bovis* bacillus Calmette-Guerin. J. Immunol. **170:**463–469.
11. **Geginat, J., F. Sallusto, and A. Lanzavecchia.** 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. J. Exp. Med. **194:**1711–1719.
12. **Hage, C. A., K. A. Mohammed, and V. B. Antony.** 2004. Pathogenesis of pleural infection. Respirology **9:**12–15.
13. **Jalapathy, K. V., C. Prabha, and S. D. Das.** 2004. Correlates of protective immune response in tuberculous pleuritis. FEMS Immunol. Med. Microbiol. **40:**139–145.
14. **Kroegel, C., and V. B. Antony.** 1997. Immunobiology of pleural inflammation: potential implications for pathogenesis, diagnosis and therapy. Eur. Respir. J. **10:**2411–2418.
15. **Ley, K., and G. S. Kansas.** 2004. Selectins in T-cell recruitment to nonlymphoid tissues and sites of inflammation. Nat. Rev. Immunol. **4:**325–335.
16. **Menezes-de-Lima-Júnior, O., and M das G. Henriques.** 1997. Mechanisms of cell accumulation induced by Mycobacterium bovis BCG. Mem. Inst. Oswaldo Cruz **92:**227–232.
17. **Menezes-de-Lima-Júnior, O., E. Werneck-Barroso, R. S. B. Cordeiro, and M. G. M. O. Henriques.** 1997. Effects of inhibitors of inflammatory mediators and cytokines on eosinophil and neutrophil accumulation induced by *Mycobacterium bovis* bacillus Calmette-Guérin in mouse pleurisy. J. Leukoc. Biol. **62:**778–785.
18. **Mohammed, K. A., N. Nasreen, J. Hardwick, R. D. Van Horn, K. L. Sanders, and V. B. Antony.** 2003. Mycobacteria induces pleural mesothelial permeability by down-regulating beta-catenin expression. Lung **181:**57–66.
19. **Mohammed, K. A., N. Nasreen, M. J. Ward, and V. B. Antony.** 1999. Helper T cell type 1 and 2 cytokines regulate C-C chemokine expression in mouse pleural mesothelial cells. Am. J. Respir. Crit. Care Med. **159:**1653–1659.
20. **Moura, A. C., P. S. Leonardo, M. G. Henriques, and R. S. Cordeiro.** 1999. Opposite effects of *M. leprae* or *M. bovis* BCG delipidation on cellular accumulation into mouse pleural cavity. Distinct accomplishment of mycobacterial lipids in vivo. Inflamm. Res. **48:**308–313.
21. **Nasreen, N., K. A. Mohammed, M. J. Ward, and V. B. Antony.** 1999. *Mycobacterium*-induced transmesothelial migration of monocytes into pleural space: role of intercellular adhesion molecule-1 in tuberculous pleurisy. J. Infect. Dis. **180:**1616–1623.
22. **Novelli, F., P. Bernabei, L. Ozmen, L. Rigamonti, A. Allione, S. Pestka, G. Garotta, and G. Forni.** 1996. Switching on of the proliferation or apoptosis of activated human T lymphocytes by IFN-gamma is correlated with the differential expression of the alpha- and beta-chains of its receptor. J. Immunol. **157:**1935–1943.
23. **Penido, C., A. Vieira-de-Abreu, M. T. Bozza, H. C. Castro-Faria-Neto, and P. T. Bozza.** 2003. Role of monocyte chemotactic protein-1/CC chemokine ligand 2 on γδ T lymphocyte trafficking during inflammation induced by lipopolysaccharide or *Mycobacterium bovis* bacille Calmette-Guerin. J. Immunol. **171:**6788–6794.
24. **Rothaeusler, K., and N. Baumgarth.** 2006. Evaluation of intranuclear BrdU detection procedures for use in multicolor flow cytometry. Cytometry A **69:**249–259.
25. **Schreiber, T., S. Ehlers, S. Aly, A. Hölscher, S. Hartmann, M. Lipp, J. B. Lowe, and C. Hölscher.** 2006. Selectin ligand-independent priming and maintenance of T cell immunity during airborne tuberculosis. J. Immunol. **176:**1131–1140.
26. **Spangrude, G. J., B. A. Braaten, and R. A. Daynes.** 1984. Molecular mechanisms of lymphocyte extravasation. I. Studies of two selective inhibitors of lymphocyte recirculation. J. Immunol. **132:**354–362.
27. Reference deleted.
28. **Tietz, W., Y. Allemand, E. Borges, D. von Laer, R. Hallmann, D. Vestweber, and A. Hamann.** 1998. CD41 T cells migrate into inflamed skin only if they express ligands for E- and P-selectin1. J. Immunol. **161:**963–970.
29. **Waters, W. R., T. E. Rahner, M. V. Palmer, D. Cheng, B. J. Nonnecke, and D. L. Whipple.** 2003. Expression of L-selectin (CD62L), CD44, and CD25 on activated bovine T cells. Infect. Immun. **71:**317–326.
30. **Werneck-Barroso, E., A. C. Moura, M. M. Monteiro, O. Menezes de Lima Júnior, M. N. de Meirelles, and M. G. Henriques.** 2000. Distinct ability to accumulate eosinophils during the inflammatory cellular response to M. bovis BCG in the mouse pleural cavity. Inflamm. Res. **49:**206–213.
31. **World Health Organization.** 1998. Laboratory services in tuberculosis control. Part III. Culture. WHO, Geneva, Switzerland.
32. **Xing, Z., J. Wang, K. Croitoru, and J. Wakeham.** 1998. Protection by CD4 or CD8 T cells against pulmonary *Mycobacterium bovis* bacillus Calmette-Guerin infection. Infect. Immun. **66:**5537–5542.
33. **Zhao, Z., K. Furukawa, S. Fukumoto, M. Okada, R. Furugen, H. Miyazaki, K. Takamiya, S. Aizawa, H. Shiku, T. Matsuyama, and K. Furukawa.** 1999. Attenuation of interleukin 2 signal in the spleen cells of complex ganglioside-lacking mice. J. Biol. Chem. **274:**13744–13747.

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